Review

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Hepatitis C virus resistance to protease inhibitors

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Recent advances in molecular biology have led to the development of novel small molecules that target specific viral proteins of the hepatitis C virus (HCV) life cycle. These drugs, collectively termed directly acting antivirals (DAA) against HCV, include a range of non-structural (NS) 3/NS4A protease, NS5B polymerase, and NS5A inhibitors at various stages of clinical development. The rapid replication rate of HCV, along with the low fidelity of its polymerase, gives rise to generations of mutations throughout the viral genome resulting in remarkable sequence variation in the HCV population, known as a quasispecies. The efficacy of DAAs is limited by the presence of those mutations that give rise to amino-acid substitutions within the targeted protein, and that affect the viral sensitivity to these compounds. Thus, due to the high genetic variability of HCV, variants with reduced susceptibility to DAA can occur naturally even before treatment begins, but usually at low levels. Not surprisingly then, these changes are selected in patients either breaking through or not responding to potent DAA treatment. *In vitro* or *in vivo*, six major position mutations in the NS3 HCV protease (36, 54, 155, 156, 168, and 170) have now been reported associated with different levels of resistance. The amino acid composition at several of the drug resistance sites can vary between the HCV genotypes/subtypes, resulting in different consensus amino acids leading to a reduction in replicative fitness as well as reduced DAA sensitivity. Different amino acid diversity profiles for HCV genotypes/subtypes suggest differences in the position/type of immune escape and drug resistance mutations. Also, different pathways of resistance profiles based on the chemical scaffold (linear or macrocyclic) of the protease inhibitors have been described. This review first describes how resistance to a protease inhibitor can develop and then provides an overview of the mechanism of how particular mutations confer varying levels of resistance to protease inhibitor, which have been identified and characterized using both genotypic and phenotypic tools. Future potential therapeutic strategies to assist patients who do develop resistance to protease inhibitors are also outlined. The challenge developing new HCV protease inhibitors should take into consideration not only the antiviral potency of the drugs, the occurrence and importance of side effects, the frequency of oral administration, but also the resistance profiles of these agents.

Keywords: Hepatitis C virus; Protease inhibitors; Direct Antiviral Agents; HCV drug resistance; Variants; HCV NS3 protease resistance; Quasispecies.
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Introduction

Increasing the sustained virological response (SVR) in patients with chronic hepatitis C (CHC) is becoming a reality with the preclinical and clinical development of more than 50 new specific drugs active against HCV [1–3]. These drugs, collectively named directly acting antivirals (DAAs), are under advanced clinical trials to be used either as an adjunct to current standard of care (SOC) therapy in triple, quadruple, or multiple combinations of HCV antiviral therapies or in various combinations of DAAs [4–10]. The encouraging data from early clinical trials of HCV-specific protease and polymerase inhibitors have been tempered by subsequent studies demonstrating the early selection of resistant variants [11,12]. Drug resistance can be an inevitable outcome using antiviral agents because of the high adaptability of the hepatitis C virus (HCV) and the failure to maintain a high pressure of inhibition [13–17]. Not surprisingly, resistance has been demonstrated for all classes of DAAs in more advanced clinical testing for NS3/4A protease inhibitors and *in vitro* for nucleoside NS5B polymerase inhibitors as well as the non-nucleoside NS5B polymerase inhibitors [16,18]. Genotypic sequence analysis of HCV RNA supports the concept that these early variants are pre-existing species, present before initiation of therapy due to the high genome replication rates and high intrinsic RNA polymerase error rates, rather than being selected for *de novo* [19–24]. Fortunately, analysis of these variants in replicon assays has suggested that the substitutions associated with the greatest resistance are also associated with reduced viral fitness [16,17,25,26].

The first generation of protease inhibitors (telaprevir and boceprevir) are in the final stages of clinical development and it is now important to better understand mechanisms of cross-resistance (when a mutation that mediates resistance to one drug also confers resistance to another in the same class) [4,27,28]. The second generation of protease inhibitors (PI) should result in compounds with more powerful antiviral activity but unfortunately, with still some level of cross resistance mutations within the class [29–40]. The incidence and prevalence of resistance in the different clinical trials should focus on the future use of the

PIs and the role of potential combination therapies. Patterns of resistance with PIs are described in this review and it is essential that clinical practice guidelines be developed in order to optimize and interpret resistance algorithms so that DAA therapy can be tailored accordingly. Characterizing resistance to DAA in clinical trials is essential for understanding how to optimize the use of a drug regimen and to provide insights into strategies aimed at maximizing SVR rates and thereby minimizing resistance.

Causes of antiviral drug resistance in the context of chronic hepatitis C

Antiviral drug resistance reflects reduced susceptibility of a virus to the inhibitory effect of a drug and results from a process of adaptive mutations under DAA therapy. A resistance mutation (nucleotide change) or substitution (amino acid change) is a change observed for an emerging variant which significantly increases in proportion, on a population basis, during DAA administration. The risk of selection of drug resistance is related to the incomplete suppression of viral replication. Several factors affect this phenomenon including: high replication rates, low fidelity of the viral polymerase, selective pressure of the drug, role of replication space (liver turnover), fitness of the escape variant and genetic barrier of the drug.

Dynamics of HCV variants: magnitude and rate of virus replication plus fidelity of the viral RNA polymerase

Because of the high mutation rate of the HCV polymerase (10^{-3} to 10^{-5}) misincorporations per nucleotide copied and the high viral production rates *in vivo* (approximately 10^{12} viruses per patient per day [41]), it can be assumed that HCV exists as a diverse population of non identical but closely related viral genomes referred to as a quasispecies [42,43]. A viral quasispecies is characterized by a dominant nucleotide sequence, or master sequence, and a surrounding mutant spectrum, which can harbor minority subpopulations [44].

The detailed study of the dynamics of viral variants present in a quasispecies population has long been hampered by the lack of sensitive sequencing methods. The recent development of deep sequencing technologies may facilitate a better understanding of the genetic composition and natural evolution of viral quasispecies in the presence of antiviral drugs [45].

The diversity of the viral variants present in an infected individual facilitates the adaptation of the quasispecies to external pressure, such as antiviral treatment, thereby improving the survival chances of the population. The speed of such adaptation depends mainly on the turnover of the viral nucleic acid acting as a source of new viral genomes [45]. Perelson and colleagues demonstrated by calculating the rates of generation of various HCV variants, that the preexistence and selection of drug-resistant variants is inevitable, and estimated the number of substitutions a combination of direct antivirals would need to achieve in order to adequately control HCV replication [17]. Thus, prior to antiviral therapy, because of this quasispecies pool, variants carrying single and double mutations potentially associated with DAA resistance will pre-exist, while three or four mutations should present sufficient genetic barrier [16,17,25,26].

Selective pressure of the drug

The probability of a mutation associated with drug resistance being selected out during therapy depends on the efficacy of that drug and this probability has been depicted graphically as a bell-shaped curve [46]. Hence, a drug with low antiviral activity does not exert significant selection pressure on the virus and the risk of drug resistance emerging is not high. Conversely, complete suppression of viral replication allows almost no opportunity for resistance to emerge because as highlighted above, mutagenesis is replication dependent [47]. Unfortunately, since monotherapies exert varying degrees of antiviral activity directed at one single target site, they result in the highest probability of selecting for drug resistance [11,48]. The ideal treatment regimen exerts antiviral activity targeted at different sites in the viral life cycle to reduce the risk of significantly selecting drug-resistant quasispecies. Not surprisingly, resistance emerges when replication occurs in the presence of drug-selection pressure. Another known factor of emerging drug resistance variant is the poor compliance of patients which should be taken into consideration. Especially, since there will be triple therapy with the protease inhibitor (three times a day), ribavirin (two times a day) and pegylated interferon (once a week).

Amount of replication space in the liver

Replication space for a virus can be described as the potential of the liver (hepatocytes) to accommodate new transcriptional templates for that virus. This then indicates that the eventual takeover by an emerging escape variant is dependent upon the loss of the original wild-type virus. This would be governed by factors such as replication fitness as well as the turnover, the number of infected hepatocytes and proliferation of hepatocytes. Hepatocyte turnover in the normal liver is slow, displaying a typical half-life of over 100 days [49], but this can be reduced to less than 10 days in the setting of increased necroinflammatory activity or associated toxicity [49]. Thus, in a fully infected liver, synthesis of new HCV transcriptional templates could only occur if uninfected cells are generated by normal growth within the liver (from hepatocyte proliferation and turnover) or there is loss of wild-type dominant viral replication transcriptional templates from the existing HCV-infected hepatocytes [16].

Replication fitness of the drug resistant virus

Replication fitness has been defined as the ability to produce offspring in the setting of natural selection [47]. This is not a yield measurement of viral replication and is usually measured in *in vitro* coinfection competition assays. Several of these assays have been developed for HCV. Although not a measure of resistance per se, the viral fitness (replicative capacity) of resistant variants is an important factor, with implications for the emergence of resistance. Viral fitness can also be defined as the replication efficiency of resistant variants, in a ratio to the replication efficiency of wild-type (WT) HCV. Because the most commonly available infectious virus system is based on a genotype 2a virus (many new HCV drugs target genotype 1), the replication capacity of HCV variants is typically assessed *in vitro* using a transient replicon system, or can be examined by comparing colony formation efficiency of the mutant replicon RNA with that of WT variants in co-culture growth competition assays [50]. Moreover replicon-based assays might underestimate the loss of fitness

caused by PI-resistance mutations, because some mutations in the NS3 protease domain specifically impair late steps in the viral life cycle that involve intracellular assembly of infectious virus.

Protease inhibitor-resistant HCV mutants have been recently shown to have reduced fitness as a consequence of impaired production of infectious virions [51]. Additionally, fitness has been determined *in vivo* by using HCV RNA levels and clonal sequencing to calculate the frequency of a given variant over time after the end of dosing to assess the growth rate compared with WT in the absence of drug-selection pressure [25]. The kinetics of selection of different resistant variants suggests that these variants are present before dosing at different levels, depending on their fitness compared with WT [45]. A detailed examination of intra-individual variation using cloning techniques or next generation sequencing approaches such as ultradeep pyro-sequencing, may provide additional information regarding the evolution and importance of low-level drug resistance variants and allow the identification of clinically relevant new variants [52,53].

Genetic barrier

The genetic barrier is the threshold probability that the virus will mutate and escape from the selective action of the drug. It can also be defined as the number of mutations needed for the development of primary antiviral drug resistance.

As the number of mutations required for resistance increases, the genetic barrier increases [54]. Thus, the genetic barrier can be viewed as the number of mutations required for a virus to become drug resistant and the probability to select mutation(s) in the presence of the drug. Fig. 1 illustrates how the rapid appearance of HCV drug resistance results in treatment failure using monotherapy with a PI or a non-nucleoside polymerase inhibitor. Schematically, a low genetic barrier is one which involves a primary mutation that comes at a low cost to the virus (in terms of fitness) and can emerge quickly. In contrast, a higher genetic barrier is one which involves not only a primary mutation with a high cost to fitness, but also second or third mutation(s) in order to generate "complete" resistance. Furthermore, second or third site mutations may also be required in order to restore fitness of these low-fitness/high resistance variants. In the case of the PIs, the genetic barrier is influenced by the HCV subtype 1a and 1b as well. Two nucleotide changes are required to generate an aminoacid change in position 155 in subtype 1b isolates [12,55]: R155K [CGG → AAG], while only one (R155K [AGG → AAG]) is needed for subtype 1a.

In contrast with the rapid emergence of drug-resistant variants to the PIs and non-nucleoside polymerase inhibitors, there is no evidence of drug resistance emerging to the current nucleoside analog polymerase inhibitors, even when they are used as monotherapy. Lack of resistance to these nucleoside analog inhibitors may be due to significantly reduced replication capacity of the resistant variants (S282T of NS-5B) or different binding sites of nucleoside and non-nucleoside inhibitors [56]. Indeed, the nucleoside analog polymerase inhibitors seem to have a higher genetic barrier to resistance than either the PI or non-nucleoside polymerase inhibitors at least in the replicon system, highlighting the potential clinical importance of these nucleoside analog polymerase inhibitors in future HCV therapies [35,55,57].

Other factors

Host factors affecting antiviral therapy include previous drug experience, compliance, host genetic factors (e.g. inborn errors

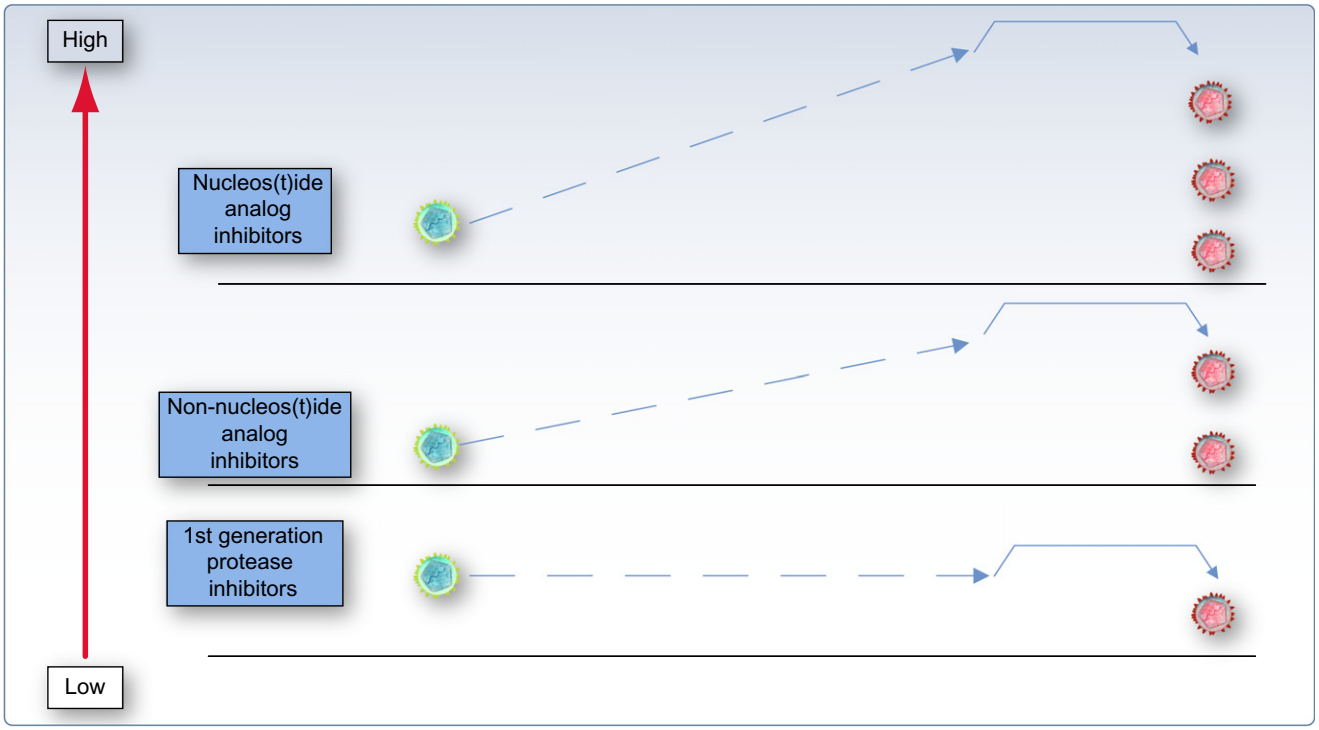


Fig. 1. Genetic barriers (low to high) for HCV Direct Antiviral Agents. The genetic barrier is defined as the threshold probability that the virus will mutate and escape from the selective action of the drug. It can also be defined as the number of mutations required for a virus to become drug resistant. Low barrier: one mutation = low cost to fitness, can emerge quickly in contrast to Intermediate or High barrier: one mutation = high cost to fitness and may require second or third mutation(s) to increase fitness.

of metabolism and strength of interferon system responses) [58] and the ability to efficiently convert the DAA to its active metabolite (if they are acting as a prodrug) via several intracellular phosphorylations (for example, in the case of nucleoside analogs and their reliance on intrahepatic salvage enzymes) [59]. In addition, there are sequestered sites/sanctuaries of viral replication that may not be accessible to the antiviral agent. Host genetic factors have been recently associated with the reponse to antiviral therapy: a single nucleotide polymorphisms (SNPs) near the interleukin (IL)-28B (IFN-lambda) region was found associated with the treatment response [58]. Although all of the identified variants lie in or near the IL-28B gene, none of them has an obvious effect on the function of this gene [60].

Kinetics of resistant mutants and viral dynamics model

From the preceding discussion, it is now possible to consider a resistance mutation as a mutation observed in a variant population which increases in relative proportion to the original WT during drug administration. A model prediction of the mutant frequency and viral load decay profiles after drug administration has recently been reported [16,45]. During telaprevir dosing, the overall viral load initially declined as the WT was inhibited and replication space available to variants increased, allowing pre-existing variants with sufficient on-dosing fitness to emerge. The increase in replication space and the on-dosing fitness of variants were the primary determinants of HCV RNA rebound during telaprevir dosing, with negligible contribution from mutations during treatment. The finding of variants prior to dosing and within a week on-treatment in other studies does suggest that the variants contributing to virologic rebound in this study were likely to pre-exist [11]. The pre-existence of variants is supported by the modeling results; had they not pre-existed, calculations based on the HCV mutation rate, replication rate, and HCV RNA level at baseline would indicate that rebound is delayed. A mathematical model of the monitoring and characterization of the dynamics of sensitive and resistant viral populations demonstrated a biphasic decrease of both drug-sensitive and drug-resistant virions after drug dosing [6,7]. The frequency of the resistant variant undergoes a substantial increase within a few days following drug administration, consistent with observations in clinical studies. The high frequency of drug-resistant variants observed early in therapy may simply be a consequence of a rapid and profound decline of wild-type virus, unveiling preexisting HCV variants [16]. Over a longer time interval, the tradeoff between the reduced susceptibility to PIs and resistance-associated fitness loss will probably determine whether such HCV variants can dominate the virus population. This balance is affected by several inter-related factors as a change in the replicative environment occurs.

Small-molecule inhibitors of viral enzymes

The development of DAA that block essential viral enzymes represents a rational and straightforward approach to developing new anti-HCV agents [61]. Although all HCV enzymes are, in theory, equally appropriate for therapeutic intervention, the NS3-4A serine protease, the NS5B RNA polymerase and the NS5A protein have emerged as the most promising of targets so far. A number of competitive inhibitors of the NS3 protease as well as nucleoside and non-nucleoside inhibitors of the NS5B polymerase and NS5A inhibitors are being developed [62]. The efficacy shown by NS3 serine protease and the NS5B RNA-dependent RNA polymerase inhibitors in recent proof-of-concept clinical trials has validated the effort spent in search of more clinical candidates thereby renewing interest in this area.

HCV NS3 protease

The NS3 protein is a multifunctional protein that harbors a serine protease located in the N-terminal one-third (189aa) which is involved in cis-cleavage of the NS3-NS4A site. The NS3 forms a heterodimer with NS4A and results in the subsequent downstream cleavage of the NS4A/4B, NS4B/5A, and NS5A/B junctions in the nonstructural region of the polyprotein. The C-terminal two-thirds of NS3 exhibits NTPase/RNA helicase activity (442aa). HCV NS3-4A serine protease is a member of the chymotrypsin serine protease family and is a heterodimeric protease, comprising the amino-terminal domain of the NS3 protein and the small NS4A cofactor. Its activity is essential for the generation of components of the viral RNA replication complex. Analysis of the X-ray crystal structure of the enzyme reveals a shallow catalytic site located on the surface of the protein, which has made development of inhibitors a formidable task (Fig. 2). Attempts to discover leads by traditional approaches of screening compound libraries have proved futile and, therefore, researchers adopted a rational structure-based drug design approach. Analysis of the X-ray structure of the NS3 protease revealed close proximity of S1–S3 and S2–S4 pockets. A number of novel approaches have been used to design preorganized, depeptidized macrocyclic inhibitors linking the P2–P4 groups and P1–P3 residues.

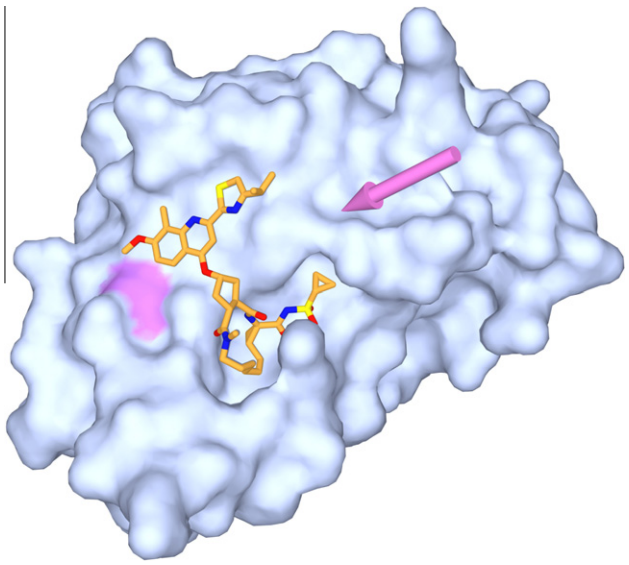


Fig. 2. The active site of HCV NS3 protease is shallow and located on the surface of the enzyme and shown in this diagram with an example of a protease inhibitor TMC-435 (Crystal structure of wild-type HCV NS3-4A with TMC-435 (PDB-ID: 3KEE)).

More recently, HCV NS3-4A protein was recognized as a multifunctional enzyme taking part in host immune evasion [63]. NS3-4A proteolytic activity is directly incriminated in this immune host evasion by blocking the activation of interferon regulatory factor 3 (IRF-3) via cleavage of TRIF and also CARDIF [64,65]. Theoretically, a potent HCV NS3-4A PI would potentially have an additive or even synergistic impact on controlling HCV replication and reducing persistence. Thus, the HCV NS3-4A PIs act at one of the middle steps of the HCV replication cycle (HCV polyprotein processing), and also at an initial step during the triggering of the host response to HCV infection. Therefore, the NS3 represents a dual therapeutic target, the inhibition of which should not only block viral replication but also restore hepatocyte innate immune control of HCV replication. This finding may explain the exceptional antiviral activity found *in vitro* and *in vivo*, with potent HCV NS3-4A PIs [66].

Protease inhibitors

For the PI molecules currently in clinical trials, the chemical scaffold of the different HCV PIs can be divided into two classes: macrocyclic compounds (MK 7009, TMC435350, ITMN191, BI12202, GS-9256, ABT 450, and BMS650032, BMS 791325) and linear ketoamids (VX 950, SCH503034, SCH567312) (Table 1). The antiviral activity of these PIs is mainly relevant

Table 1. Description of the protease inhibitors in clinical phase. *In vitro* Huh-7 replicon wild-type HCV 1b average range of antiviral activity (nM), pharmaceutical company, chemical scaffold and type of structure (linear or macrocyclic). Non available structure (NA). *Putative structure.

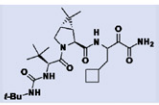
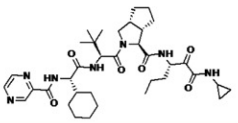
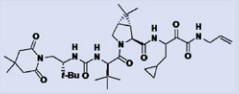
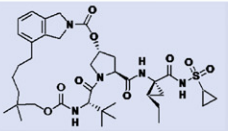
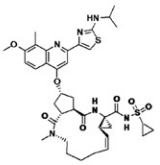
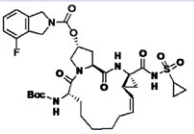
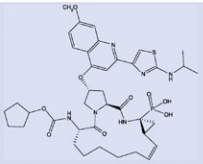
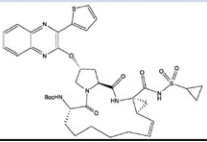
Agent	Pharmaceutical company	Antiviral activity Huh-7 1b (EC 50 nM)*	Phase of development	Type	Chemical scaffold
Telaprevir (Vx-950)	Vertex/Janssen	300-400	Filed	Linear	
Boceprevir (SCH 503034)	Merck	400-500	Filed	Linear	
Narlaprevir (SCH 900518)	Merck	50-100	Phase II	Linear	
BI 201335	Boehringer	1-10	Phase II	Linear	NA
Vaniprevir (MK-7009)	Merck	1-10	Phase II	Macrocyclic	
TMC 435350	Janssen/J and J	1-10	Phase II	Macrocyclic	
BMS-791325	BMS	1-10	Phase II	Macrocyclic	NA
Danoprevir (IMN191/RG7227)	Roche	1-10	Phase II	Macrocyclic	
Ciluprevir (GS-9256*)	Gilead	10-50	Phase II	Macrocyclic	
Enanta/ABT-450*	Abbott	1-10	Phase II	Macrocyclic	

Table 2. Amino acid positions within the NS3/4A protease associated with resistance mutations to different NS3 protease inhibitors and a cross-resistance table of different NS3 protease inhibitors based on mutations selected in patients from clinical studies and/or from *in vitro* studies. Resistance mutations of NS3 protease inhibitors with a ⩾4-fold increase in EC_{50} are shown in *gray* (Resistant) and resistance mutations described ⩽4-fold change in EC_{50}; are shown in *white* (S = susceptible) EC_{50} = 50% effective concentration (replicon HCV-1b).

	V36A/M	T54A	V55A	Q80R/K	R155K/T/Q	A156S	A156V/T	D168A/V/T/H	V170A
Telaprevir (linear)			*						*
Boceprevir (linear)							*		
SCH900518 (linear)									
BILN-2061 (macrocyclic)									
ITMN191 (macrocyclic)						*	*		
MK7009 (macrocyclic)						*			
TMC435350 (macrocyclic)									
BI-201335 (linear)									
MK5172 (macrocyclic)									
GS-9256 (macrocyclic)									
ABT 450 (macrocyclic)									
BMS-791325 (macrocyclic)									

*Mutations associated with resistance in vitro only.

to patients infected with HCV genotype 1, since the antiviral activity of these current PIs against genotype non-1 genotypes (except for genotype 2), is not as effective [67]. A number of compounds are in preclinical phases with broader activity across the genotypes and with more favorable cross-resistance profiling. This distinction has led to two different resistance profiles, with the R155 and D168 substitutions mainly found associated with the macrocyclic compounds rather than the linear compounds. Substitution at the A156, V36 and T54 are more strongly associated with resistance from the linear compounds rather than the macrocyclic compounds (Table 2). Other more class-specific mutations are found due to the contact affecting the P2–P3 link, inducing a strong steric hindrance with a modification of the P2 positioning which cannot be compensated by all the PIs and thus constitute one of the major cross resistance mutations (Fig. 3) [68].

Molecular modeling of protease mutations with HCV protease inhibitors

Cross resistance mutations between linear ketoamids and macrocyclic PIs: A156T and R155K

A156T is one of the major class-specific mutations that to date has been found to affect PIs; this is because of the contact affecting the P2–P3 link, inducing a strong steric hindrance with a modification of the P2 positioning which cannot be compensated with P4–S4 and P5–S5 interactions (Fig. 4) [68].

R155K is the other class-specific mutation. Molecular modeling of the complex with a linear ketoamid or a macrocyclic indicate that the alkyl side chain of the arginine has several Van der Walls (vdW) interactions and so the substitution by lysine at 155 has an effect on the P2-substituted quinoline moiety of macrocyclics and induces a buried salt bridge formed by R155-D168. Therefore, mutations at either position 155 or 168 could severely disrupt this salt bridge and affect its interaction with both macrocyclic protease inhibitors resulting in resistance. In contrast, linear-ketoamide inhibitors, like VX-950 and SCH-503034, do not possess a large P2 moiety that can force the formation of a buried R155-D168 salt bridge. Both R155 and D168 side chains adopt solvent-exposed conformations with all five HCV PIs tested (VX-950, SCH-503034, ITMN-191, MK-7009, and TMC-435350). The linear-ketoamide inhibitors make only a few contacts with R155 and D168 side chains, which is consistent with low-level resistance for R155K mutations observed (Fig. 5).

V36M and T54A

V36M and T54A substitutions, individually, confer low level resistance to boceprevir and telaprevir (fold resistance (FR) <10), but confer intermediate resistance to telaprevir if associated together (V36M + T54A, FR = 20), and high resistance level if associated with A156T or R155K (telaprevir, FR >62) [25,69]. Therefore, V36M and T54A increase in a synergistic way (potentiate), the resistance effect associated with the mutation R155K or A156T. The buried residues Val-36 and Thr-54 are located near the protease catalytic domain of HCV NS3-4A but are not close to

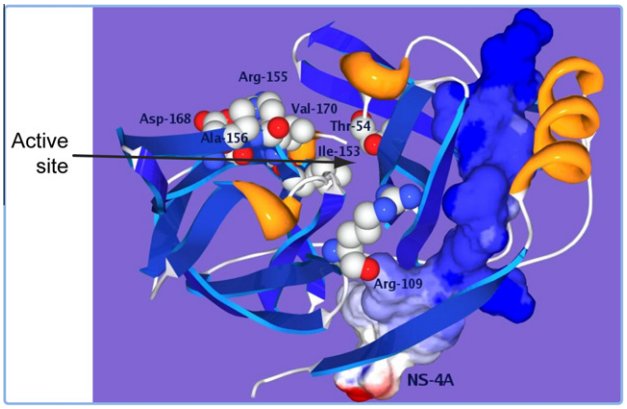


Fig. 3. Three-dimensional overview of HCV NS-3. Location of PI-resistant mutations is shown. Both A156 and R155 are located in the active site. T54 and V36 are close to but not inside the active site and do not make direct contact with telaprevir.

where the PI boceprevir and telaprevir are located in their respective complexes with HCV NS3-NS4 protease (d >6 Å). The distance between the ketoamide warhead of both PIs and V36M and T54A, are, respectively, 11 Å and 8 Å. Thus, V36M and T54A are not in direct contact with both PIs (Fig. 6). These mutations will discriminate boceprevir and teleprevir through an indirect resistance mechanism [68]. In the wild type complex, Val-36 has van der Walls (vdW) contacts with the neighbor residue Phe-43 (d = 3.4 Å), which is in contact with Gln-41 (d = 3.7 Å), a residue in direct contact with the P1′ part of the keto-amide based PIs (d = 3.4 Å). In the case of the mutation V36M, from a beta branched (Val) to a linear amino acid (Met), the contacts between residues V36M and Phe-43 are lost, bringing more flexibility to the Phe-43 side chain. Like a domino cascade, the V36M mutation, through Phe-43, will destabilize Gln-41 side chain decreasing its hydrogen bonding with the P1′ of boceprevir or telaprevir. It is noteworthy that the mechanism of HCV NS3-4A protease inhibition by keto-amide based PIs occurs in two successive steps. First, a weak affinity with no covalent complex occurs which is then followed by the formation of a reversible covalent complex of high affinity. The Gln-41 side chain forms a hydrogen bond with the carboxamide of P1′ moiety of boceprevir and telaprevir in the reversible covalent complexes, and Gln-41 takes part equally to the positioning of the P1′ moiety during the no covalent recognition step. The domino cascade events brought by V36M substitution disrupts the hydrogen bond between Gln-41 and P1′ during the non-covalent recognition step and also during the reversible covalent step.

In the wild type boceprevir cocrystal structure, T54 forms one hydrogen bond with L44 (—OH···O═C, d = 2.8 Å, PDB-ID: 2OC8), the neighbor residue of Phe-43 and also has a Van der Waals (vdW) contact with the Phe-43 side chain [70]. Analyzing the hydrogen bond network surrounding Thr-54, the Leu-44 forms equally a hydrogen bond with the main chain of the catalytic residue Ser-139 (N—H···O═C, d = 2.9 Å). Ser-139 is a crucial residue for the proteolytic activity of NS3-4A protease (catalytic residue), but Ser-139 is also crucial for the formation of the reversible covalent complex with keto-amide based PIs like boceprevir and telaprevir. Moreover, Thr-54 forms a weak contact with the backbone of Ser-139 (d = 4.6 Å). T54A/S substitutions have been described as boceprevir and telaprevir resistance changes, indicating a probable molecular mechanism of resistance of T54A/S strains for boceprevir and telaprevir [25,71]. If just the weak contact between Thr-54 side chain and Ser-139 main chain was incriminated in the resistance, the mutation T54S may bring few perturbations because this substitution may allow Ser-54 to keep a weak contact with Ser-139. But the T54S change can remove or decrease the direct contact with Leu-44 main chain (hydrogen bond between Thr-54 side chain and Leu-44 main chain). Thereby, the mutation T54S may have more impact on Leu-44 compared to Ser-139. This mechanism would predict a higher resistance of T54A strain to boceprevir and telaprevir because of the elimination of electrostatic contacts with Ser-139 and hydrogen bonding with Leu-44. This finding has been validated by the phenotype analysis obtained by Susser et al. showing an increase in resistance from T54S to T54A for both telaprevir and boceprevir (from FR = T54S: 4, 5 to T54A: 10, 16, respectively [25]. Phenotypic analysis has revealed that boceprevir is more prone to resistance compared to teleprevir against the T54A mutation (FR = 16 vs 10, respectively). Thr-42 may be the explanation of this peculiarity. Indeed, the keto-amide P1′ of boceprevir contains a primary amide extremity allowing a double

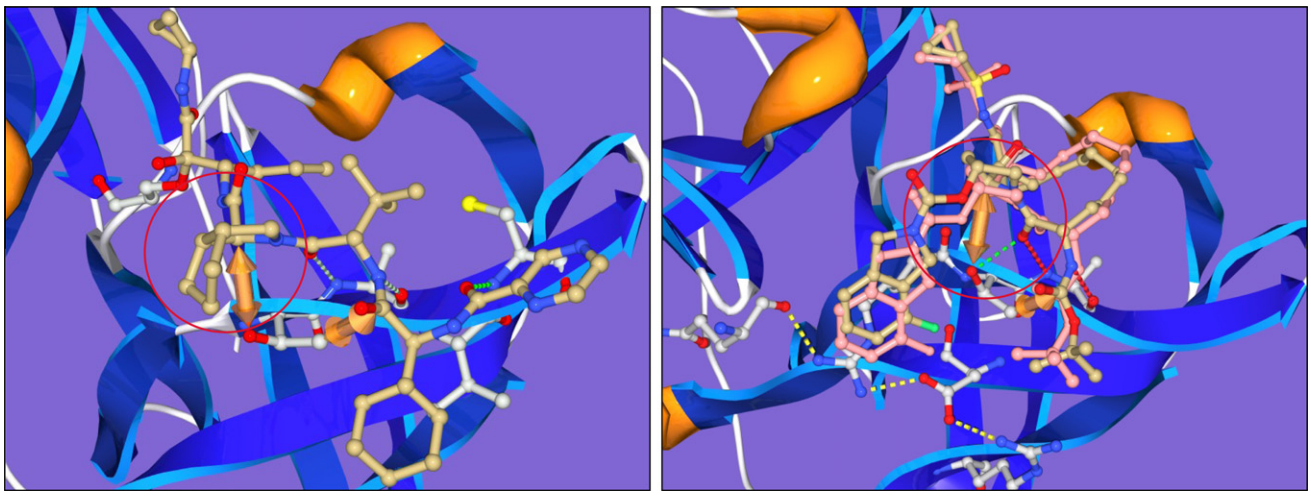


Fig. 4. (A) VX 950 and (B) TMN-191 compounds resistance mechanism/A156T. A156T is one of more class-specific mutations that are found due to the contact affecting the P2–P3 link, inducing a strong steric hindrance with a modification of the P2 positioning which cannot be compensated with P4–S4 and P5–S5 interactions.

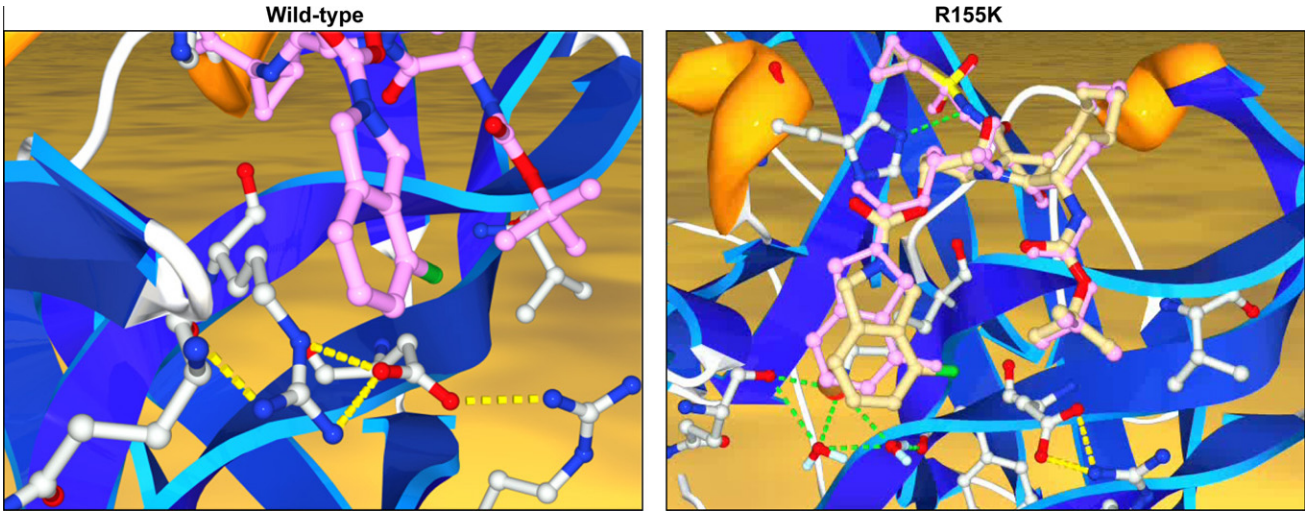


Fig. 5. ITMN191 compound resistance mechanism/R155K (WT 5A) and mutant R155K (5B). Molecular modeling of the complex with a macrocyclic (example of ITMN-191) indicates that the arginine has some Van der Walls (vdW) interactions with R155 alkyl side chain and that the substitution by the lysine in 155 on the P2 substituted quinoline moiety of macrocyclic and induces a buried salt bridge formed by R155-D168. Therefore, mutations at either position 155 or 168 could severely disrupt this salt bridge and affect its interaction with both macrocyclic protease inhibitors, resulting in resistance.

hydrogen bonding with the S1′ binding pocket. The first hydrogen bond occurs with Gln-41 side chain and the second with the main chain (C═O) of Thr-42, while telaprevir contains a secondary amide P1′ extremity (*N*-methylcyclopropyl) which allows only one hydrogen bonding with the Gln-41 side chain. Thereby, if boceprevir undergoes a double hydrogen bonding perturbation in the P1–S1′ interactions domain, boceprevir will be more prone to discrimation and resistance. This perturbation is communicated from T54A through Leu-44 and Phe-43 to Thr-42. Like V36M, the mutation T54A/S induces a domino cascade events from residue 54 to the PI ketoamide warhead. T54A/S may perturb (T54S) or abrogate (T54A) electrostatic contact with Ser-139 (perturbation of the reversible covalent complex) but equally hydrogen bonding with Leu-44 and vdW contact with Phe-43. This perturbation will drive through Phe-43, Thr-42 and Gln-41 to the destabilisation of the interactions occurring between S1 binding pocket and keto-amide P1′ moiety. It is likely that the T54S mutation, which introduces a cavity in the interior of the protease, could affect the S139 position and conformation, thereby impacting the interaction of S139 with this class of inhibitor. On the other hand, the T54S mutation does not significantly affect macrocyclic inhibitors, since they do not rely on covalent interactions with S139 [72].

D168A/V

The D168A/V changes are selected *in vitro* and *in vivo* and are commonly associated with macrocyclic based PI dosing, like TMC-435, ITMN-191, MK-7009 [71]. However, this macrocyclic clustering is not the best unifying hypothesis, but instead may be related to PIs containing a large P2, such as BILN-2061, TMC-435, ITMN-191, MK-7009 (large P2 PIs) [72]. Molecular modeling of BILN-2061 complexes [68] and structural analysis of the co-crystal structure of NS3-4A protease complexed with TMC-435 (as well as ITMN-191, PDB-ID 3M5L) has shown that R155 adopts an extended conformation (TMC-435, PDB-ID: 3KEE) compared to the apo structure of NS3-4A protease or co-crystal structures with PIs having small P2, like boceprevir

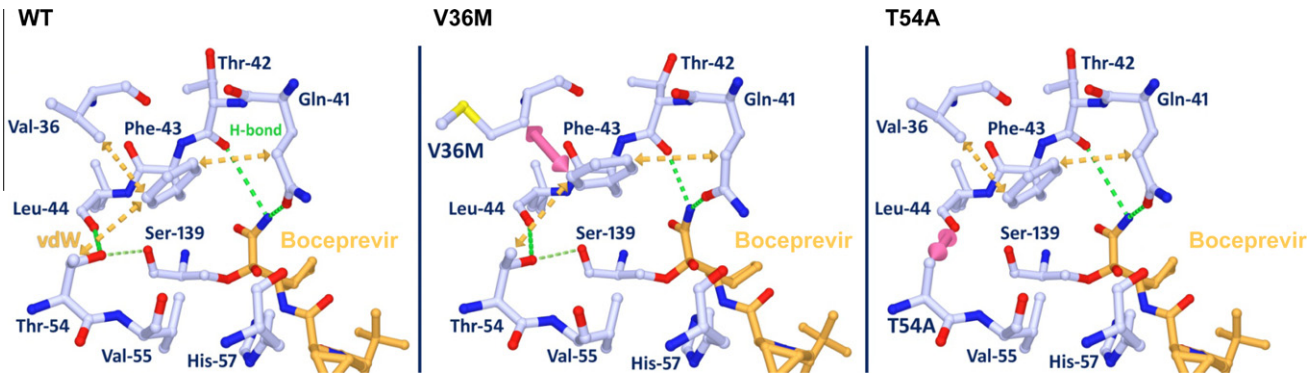


Fig. 6. WT: crystal structure of wild type HCV NS3-4A with boceprevir (PDB-ID: 2OC8). V36M: model of boceprevir in complex with V36M HCV NS3-4A (from crystal structure of V36M strain of HCV NS3-4A, PDB-ID: 2QV1). T54A: model of boceprevir binding to HCV NS3-4A T54A strain (obtained by mutation of Thr-54 to Ala from wild type crystal structure using GenMol software). Hydrogen bonds are depicted by green dashed, van der Walls contact by beige double dashed arrow, lost of contact are depicted by double pink arrow and boceprevir in orange.

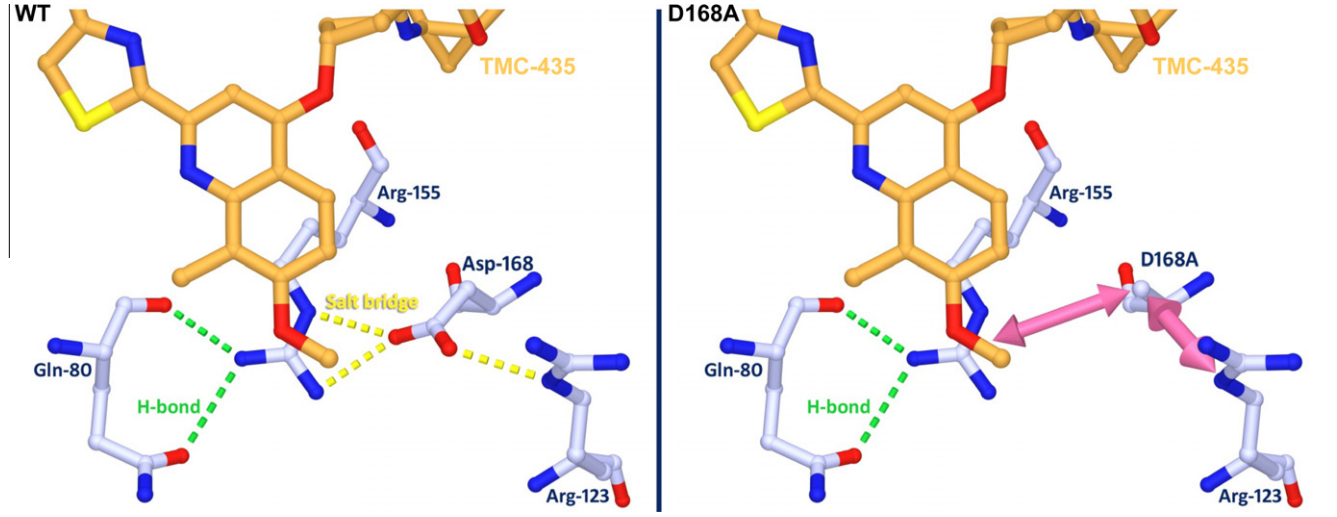


Fig. 7. WT: crystal structure of wild type HCV NS3-4A with TMC-435 (PDB-ID: 3KEE). D168A: model of TMC-435 binding to HCV NS3-4A D168A strain (obtained by mutation of Asp-168 to Ala from wild type crystal structure 3KEE using GenMol software). Hydrogen bonds are depicted by green dashes, salt bridge by yellow dashes, loss of contact (salt bridges) are depicted by double pink arrow and TMC-435 are depicted in orange.

(PDB-ID: 2OC8) as well as boceprevir analogs (PDB-ID: 2OBO, 2OCO) and telaprevir analogs (PDB-ID: 2P59), pdb-ID: 3KEE) [73] (Fig. 7). This extended R155 conformation optimizes vdW contacts between P2 and S2 and more importantly allows electronic π-stacking interactions between P2 subunit and S2 binding pocket. In both types of structures (large and small P2 PIs) D168 forms strong salt bridges with R123 from S4 binding pocket and R155 from S2 binding pocket. Therefore, D168 links S2 to S4 binding pocket through strong salt bridges (Fig. 2). For large P2 PIs like TMC-435 or ITMN-191, D168 fixes the guanidinium extremity system of R155 (taking part of S2) toward the P2 aromatic moiety of PI: indoline like for BILN-2061, TMC-435, or isoindoline like for ITMN-191 and MK-7009. The substitutions D168A/V replace the acidic negatively charged residue Asp to an apolar and hydrophobic residue Ala or Val and abrogate the key structural salt bridges between residue 155 and 168. Losing this structural anchoring, R155 will gain more flexibility and conformational freedom, decreasing its interactions with the large P2 of PI. This mechanism is in agreement with the resistance profiles of TMC-435, ITMN-191, and MK-7009 showing *in vivo* the selection of D168 mutations (mainly D168A/V), and also during the selection of BILN-2061 drug resistance *in vitro*.

HCV drug monitoring resistance tools

Information on patterns of resistance to- and cross-resistance between antiviral agents is increasingly available and may be important for decisions on how to combine drugs to achieve an optimum antiviral effect. Two complementary methods are used to characterize viral resistance: genotypic and phenotypic assays. Data from the clinical trials carried out so far, have indicated that sensitive methods should be adopted in order to assess HCV drug resistance. The use of genotypic-resistance analysis to assist therapeutic decision-making in patients using PCR-sequencing, which is the reference method, provide insight into strategies aimed at maximizing SVR rates and thereby minimizing resistance. However, there are no commercial assays available yet to characterize DAA resistance that can be used in routine clinical practice.

Genotypic (sequence analysis) assays

Genotypic analysis of HCV enables the identification of individual and combinations of nucleotide substitutions that are known to confer resistance to specific antiviral agents. Initial characterization of the resistance profile for a drug requires comparing viral sequences before, during, and after treatment to detect changes from baseline (pre-treatment) that could occur with a particular drug treatment; this could help to "individualize" drug combinations in order to attain maximum virus suppression thereby avoiding treatment failure [12]. A general understanding of fundamental assay technology, protocol feasibility, data interpretation, and sources of data variation all play a role in the selection of the optimal genotyping assay for the clinical microbiology/virology laboratory. Identification of the limitations of each assay is important for the clinician to appropriately interpret and apply test results to patient care (Table 3). The detection of viral resistance depends on the sensitivity of the assay [5]. For example, although population-based PCR sequencing methods are relatively simple to conduct and most frequently used, they cannot determine linkage between different mutations in a single variant, or detects variants with mutations that are present in less than 15–25% of the population [52]. The level of resistant variants present before treatment is initiated will most likely be below this limit of detection and can only be determined using ultrasensitive methods including clonal sequencing, ultra-deep sequencing or the TaqMan mismatch amplification mutation assay (TaqMAAMA). This latter assay can detect and quantify known minor resistant variants of HCV and has been shown to correlate well with clonal sequencing results. TaqMAMA was linear over a wide range of mutant levels (0.01–100%), and could detect consistently variants at ~0.1% level [53]. The assay was highly reproducible, with a coefficient of variance of approximately 10–30%. However, the assay also has limitations

Table 3. Diagnostic tools used for minor variants determination: description, principle, advantage, and drawback.

	Clonal sequencing	Single genome sequencing	Allele specific PCR	Ultradeep sequencing
Principle	Molecular cloning Sequencing of numerous clones	Limits dilution (1 single genome) Numerous PCR and sequence	Mismatch amplification mutation assay	Multiples short sequences analyzed 200 to 400 nt
Sensitivity	>10% (1%:100 clones) 0.1%:1000 clones	2%	0.03-0.2%	0.05-1%
Advantages	Reference method All mutations detected Double mutant detected	All mutations detected Double mutant detected	Sensibility +++ Easy to perform Possible quantitation Low cost	Sensibility ++ Automatisation Quantitation
Drawbacks	Errors/recombination during PCR Bad sensitivity Bad NPV Nb of clones and sequences ++ Costly and hard time labour Lack of automatisation	1 single copy of DNA? Costly and hard time labour Lack of automatisation	Known position of resistance Limited number of mutations detected Bad specificity Neighbour polymorphisms few standardisation	Short sequences CV >10^4 copies/ml Availability Equipment/cost ++ Interpretation

related to the fact that HCV exists in patients as a quasispecies swarm which may result in significant sequence polymorphism near the mutation site [53]. DNA chip technologies and sequencing with microchip based technology using oligonucleotide microarrays is still in development for HCV genotyping. Additionally, given the variability of HCV and the possibility that HCV populations may evolve rapidly, not all substitutions observed on treatment should be considered drug-selected changes. Appropriate statistical tests for a particular set of sequence data plus an expert virological opinion should be employed to identify significant drug-selected substitutions as per the HIV-drug resistance interpretations [74]. The cost benefit of these assays in the context of the overall cost of the PIs, will need to be determined.

HCV phenotypic assays

Phenotypic assays assess the degree of decreased susceptibility conferred by a substitution(s) by measuring the EC50 (the effective concentration of drug required to inhibit replication by 50%) in an enzyme or replicon based assay of HCV variants for drug susceptibility *in vitro*. The fold change in sensitivity can be calculated as the EC50 value of the isolate/EC50 value of the reference strain (e.g. WT). At present, these assays cannot be used in clinical studies to monitor the drug resistance profiles due to intense labor demands, cost and time. Moreover, standard replicon assays limit the assessment of the inhibitory activities of anti-HCV compounds to a few laboratory-optimized strains and may not reflect the range of activities of a compound against the heterogeneous viral population that exists in the diverse HCV-infected community of patients [28,48,71,75,76]. Biological and/or clinical cut-offs will be necessary to interpret the clinical significance of these shifts in phenotypic fold change.

Patterns and pathways of resistance

In clinical trials with telaprevir and boceprevir, resistance associated mutations have emerged quickly (less than 15 days) in patients on monotherapy. This strongly suggests the pre-existence of quasispecies harboring drug resistance mutations at low frequencies, given their rapid appearance after commencement of drug therapy. The incidence of resistance inversely correlated to SVR which is related to the potency of the antiviral activity: the compound with the highest antiviral activity (EC50 in replicon 1–50 nM) resulted in the strongest suppression of viral replication. As highlighted above, the first generation of Linear Ketoamids (telaprevir and boceprevir) select quite similar resistance profiles with the V36 and T54 mutations whereas macrocyclic inhibitors exhibit a common pathway with D168 substitutions. The R155K and A156 T/S substitutions are cross-resistant for all the Pis [12,16,48].

Telaprevir

Variants resistant to telaprevir were identified and characterized early *in vitro* [12,16,48]. The replicon system, based on a genotype 1b isolate showed an increase in mutations in position 156 of the NS3 protease domain with the substitution of alanine to serine (A156S), valine (A156V), or threonine (A156T). Using a highly sensitive sequencing assay that detects minor populations of viral variants (>5%), mutations were identified that conferred low-level (V36M/A, T54A, or R155K/T) or high-level (A156V/T and V36/R155) resistance to telaprevir *in vitro*. In patients who had a viral rebound on telaprevir alone, the R155K/T and A156V/T variants were detected during the initial steep decline in HCV RNA. During the rebound phase, the R155K/T and A156V/T variants were replaced by V36 (M/A)/R155 (K/T) double mutant variants. As discussed above, single substitutions in R155 are described for genotype 1a (AGG → AAG), whereas in genotype

1b the variant R155T requires a double nucleotide mutation (CGG = R arginine to CCG = proline, then to ACG = threonine, probably by a telaprevir-sensitive intermediate with proline in position 155) [52]. Also the V36A variant has been only described in genotype 1a *in vivo* for similar reasons [12,16,48]. Two other second site substitutions have been observed *in vitro:* Q41R and F43S, with a lower impact on fold resistance.

In the PROVE 2 trial, breakthrough was found in 8.67% of patients and relapse in 23.94%, and low level-resistance mutants were present in 10 out of 22 patients with breakthrough and 35 out of 42 patients with relapse [77]. The interim analysis of the EXTEND Phase III (long-term follow-up of patients with chronic hepatitis C treated with telaprevir in combination with Peginterferon Alfa-2a and ribavirin) study enrolled 56 patients who failed to achieve SVR from PROVE1 ($N = 30$), PROVE2 ($N = 42$), PROVE3 ($N = 99$) and Study 107 ($N = 31$), a SVR was durable in 99%) in a median time to follow-up: 22 months after SVR (range 5–35). The main mutations observed in this study were the same as those observed in PROVE 1 and PROVE 2: namely 36, 54, 155, and 156. Interestingly, 85% of genotype 1a patients with detectable resistant variants were infected with HCV V36M + R155K. Moreover, 89% of non-SVR patients (50 out of 56) who had viral variants with reduced telaprevir susceptibility at the end of the earlier studies no longer showed evidence of resistance by the end of follow-up and the resistant variants were replaced by WT virus (median follow-up time: 25 months from end of prior study [78].

Boceprevir

In vitro studies revealed that T54A, A156S/T, V170A are associated with different levels of resistance [11]. Phase II and III clinical studies (Sprint 1 and Sprint 2) indicated that A156S/T and V36M are the most frequent substitutions observed. Substitutions of >25% of the populations were: V36M, T54S, and R155K, while the less common (5 to 25%) were T54A, V55A, R155Y, A156S, V158I, V170A, and the infrequent changes (<5%) included: V36A, V36L, and I170T [11]. In the boceprevir studies, a lead in phase combining pegylated interferon and ribavirin during the first 4 weeks and then the triple therapy was utilized, which may in theory decrease the viral load thus decreasing the risk of viral resistance. Interestingly, the mean frequency of viral variants was 14% at the end of treatment. Results from a 3 year long term follow-up of 604 patients receiving boceprevir in naive patients (SPRINT-1) and non responders (P03659, P04887) indicated that SVR persists for at least 2 years after the end of treatment in the 290 responder patients. Among the 174 patients with boceprevir associated resistance substitutions detected, 64% were R155K, 32% were T54S and 33% were V36M, with a variable time of disappearance of mutations indirectly reflecting the loss of fitness of these viral variants [79].

The newly identified substitutions V158I–V163L have been selected *in vitro* and are associated with telaprevir and boceprevir-drug resistance [80]. V158I conferrs low level resistance (as shown by a 2.5-fold increase in inhibition constant Ki to boceprevir but not to telaprevir. Structural analysis of the NS3 protease active site showed that V158 is part of the substrate binding pocket and is in direct contact with the terbutyl P3 of boceprevir. Another new variant V55A, is associated with resistance in patients treated with boceprevir and telaprevir [25].

Difference in telaprevir and boceprevir treated patients for the frequency of detection and/or resistance levels between the two drugs have been observed, and this was mainly applicable for the T54A and V170A mutations.

Clinical aspects of resistance

Definitions of response

All patients receiving antiviral treatment therapy for CHC should be closely monitored for virologic response and breakthrough and for durability of response and viral relapse after treatment has stopped. Table 4 summarizes the different treatment outcomes and response for chronic HCV infection using frequent monitoring of HCV RNA levels for prompt detection of treatment failure and resistance.

How to best prevent resistance?

Prevention of resistance is a clinical challenge and can be achieved by implementing and considering several factors: (1) using a potent antiviral drug as demonstrated by its ability to exhibit the lowest incidence and prevalence of drug resistant mutants in the clinical trials done with the current generation of protease inhibitors [25]; (2) reach high level of residual plasma and tissue concentration by plasma drug monitoring. Preclinical studies of boceprevir demonstrated the frequency of emergence of resistant mutants in relation to the dosage of boceprevir, that at the concentrations $6 \times EC_{90}$ and $12 \times EC_{90}$ the frequency of resistant cells was ~0.14 and ~0.02%, respectively; (3) Maintain good compliance of patients by implementation of programmes

Table 4. Classification of outcomes of treatment for chronic HCV infection.

RVR	Undetectable HCV RNA at week 4 of treatment
cEVR (complete EVR)	Undetectable HCV RNA at week 12 of treatment
eRVR (extended EVR)	Undetectable HCV RNA at weeks 12 and 24 of treatment
SVR	Undetectable HCV RNA at the end of treatment, and for at least 24 weeks after completion of treatment
Virological breakthrough	HCV RNA levels initially decrease (even to undetectable levels), followed by a clinical relevant increase while on treatment (usually 1- 2 $\log_{10}$), but specific conditions vary between clinical trials.
Non-response	HCV RNA levels decrease slowly and to different extents, but never become undetectable
Null response	<1 $\log_{10}$ decrease in HCV RNA levels by weeks 4 or <2 $\log_{10}$ decrease in HCV RNA levels by week 12
Partial response	≥2 $\log_{10}$ decrease in HCV RNA levels by week 12, but never undetectable
Relapse	Undetectable HCV RNA levels at completion of treatment, but becoming detectable during follow-up

promoting patient adherence to treatment and (4) by increasing the genetic barriers to escape, typically by the use of combinations of drugs. This latest point may also be consolidated by using ribavirin in triple combination therapy, since the percentage of breakthrough was less when comparing the arm without ribavirin with both boceprevir and telaprevir [6,77].

Cross resistance mutations

Cross-resistance occurs when resistance mutations are selected that are common to more than one drug within each class. This is typical for inhibitors that bind the same pocket but not necessarily for inhibitors with the same mechanism of action (see molecular mechanism section). Genotyping analyses show a largely overlapping cross-resistance profile of boceprevir and telaprevir. Two mutations, A156T and R155K are associated with resistance to all the protease inhibitors. A156T, conferring high level of resistance and associated with reduced virological fitness, is mainly selected *in vitro* but occurs to a lesser extent *in vivo*. The R155K mutation confers low level of resistance to the linear compounds but results in high level of resistance to the macrocyclic group, and is frequently found *in vivo*. This change is costly for the virus and results in a loss of fitness. It is therefore not surprising that for these two substitutions, an inverse correlation between resistance and fitness has been found: the most resistant variant had the lowest fitness.

Clinical impact of resistance to the PIs

Table 2 shows mutations that have been shown to confer resistance to the PIs at varying levels, typically measured by the fold change in EC_{50} from WT. For example, the R155K variant confers low levels of resistance (<10-fold increase in EC_{50} compared with the WT) to boceprevir and telaprevir *in vitro*, and higher levels of resistance to BILN2061 (~250 fold increase) and ITMN-191 (~70-fold increase) [20]. However, what is more relevant in the clinic than a fold change is the protein-adjusted fold change and drug exposure. Drug concentrations (C_{trough}) in a patient need to achieve levels that exceed the protein-adjusted EC_{50} value of resistant variants in order to effectively control these variants clinically. Therefore, clinical resistance will always be relative to the level of resistance conferred by a variant and the drug exposure in the patient. Characterizing the variants that arise clinically can give further insight into the drug levels that may be present in patients and the ability of the regimen to suppress both WT and resistant variants.

Both groups of PIs (macrocyclic and linear) bind at the active site of NS-3 and so there is some overlap in resistance profiles. For example, the R155K mutation has been shown to confer resistance to both linear and macrocyclic inhibitors *in vitro*, albeit at different levels [18]. Based on the different structures of macrocyclic and linear inhibitors, the selection of variants with changes at different sites outside of the active site may be expected. For example, the D168 position has been shown to be important for macrocyclic protease inhibitors, while positions V36 or M54 are important for the linear inhibitors.

Variants with decreased susceptibility to PIs are likely to pre-exist at low levels since they are typically fitness impaired and thus are rarely detected by population sequencing. However, if their fitness approximates that of WT virus they can be present at higher levels and thus can be detected more frequently in the untreated population. For example, a population sequence analysis of the NS3/4A protease in 570 treatment-naïve patients with chronic HCV infection revealed that variants that confer resistance in varying levels of protease inhibitors occur naturally at a very low frequency in the HCV-infected patient population (<1% each) [20,22]. If a more sensitive assay is used, then it can be revealed that two variants (T54A and V170A) with reduced susceptibility to boceprevir were found to exist at low levels in the viral population before boceprevir treatment was initiated (in 1 or 2 clones per 90 clones sequenced) [23].

Although the clinical impact of pre-existing viral variants on treatment outcome requires further study, their presence may not necessarily predict treatment failure. One recent analysis showed that even patients who had 100% V36M low-level telaprevir-resistant variant at baseline achieved an SVR on a telaprevir-based treatment (3 out of 4 patients with V36M achieved an SVR), suggesting that the presence of resistant variants may not necessarily preclude successful treatment [20].

Hepatitis C virus drug resistance and immune-driven adaptations

Another issue for consideration in DAA resistance is related to the overlapping selection pressures from HCV PIs and the host's immune response. Recent data have suggested that variation at the drug resistance sites can occur in the absence of the specific drug pressure, and other selection pressures such as the host's immune response may be occurring at these sites. Hence, among individuals infected with subtype 1a, 21.5%, for 1b 44.4% and for 3a 41.8% exhibited genetic variation at a known drug resistance site. Specific regions within the HCV protease (and polymerase) are under both potential HLA-driven pressure and therapy selection and six HLA-associated polymorphisms have now been identified [81]. Furthermore, because very few individuals worldwide have been exposed to DAA drugs, the drug resistance variations identified here are unlikely to be attributable to the transmission of resistant viruses from individuals previously exposed to these drugs.

The issue of "Archiving" of HCV drug-resistance substitutions

The question of the existence of compartments harboring HCV drug resistant mutants against the DAAs, as has been observed in HIV infection, requires consideration [82]. The basis for the long term persistence of PI-resistant variants mutants at the end of therapy and their potential source in different anatomic sites does need to be explored in the future. The present notion is that this reservoir does not decay because it is being continuously replenished by a low level of ongoing viral replication [83]. As such this reservoir will hamper the future use of other PI. The issue remains unresolved and requires clarification as it will impact on a number of treatment intensification studies with other classes of DAAs.

Genotype 1 subtype and HCV PI resistance

In vitro selection of drug resistance carried out in the replicon model are limited by the use of only a single HCV subtype, GT-

1b, HCV subtypes can vary by up to 25% at the nucleotide level, and this variability may lead to subtype specific differences in the resistance profiles [55]. The previously reported replicon resistance studies have been performed using GT-1b replicon cells and did not address substitutions that emerged in GT-1a-treated patients The double mutants V36M + R155K have been identified only in GT-1a infected patients treated with telaprevir and not in GT-1b infected patients [6,11,48,77]. The reasons for this have already been discussed above.

In clinical studies of telaprevir alone or in combination with pegylated interferon (with or without ribavirin), selection of resistant variants and viral breakthrough have been observed consistently more frequently in patients infected with HCV subtype 1a than subtype 1b. [11,48]. Several resistant variants were selected typically in subtype 1a- (V36M, R155K/T) or 1b- (R155Q, V170A) infected patients [11,48]. Additional results from SPRINT-1 indicated that the most common resistance mutations in genotype 1a patients not achieving SVR was V36M, T54S, and R155K, whereas mutations T54A, T54S, A156S, and V170A were identified in genotype 1b patients [8]. Differences between subtype 1a- and 1b-infected patients also were observed for macrocyclic NS3 protease inhibitors (ITMN191/R7227, BI201335) [48]. Taken together, these data highlight the need to determine the subtypes of genotype 1 in patients receiving DAAs treatment. Of note, due to the lower power of discrimination of the 5′ non coding region to distinguish the subtype 1a from 1b, we strongly underline the importance of the choice of another region as NS5b or E1 for HCV genotyping [19,84].

Combination therapies and SOC-free treatments

Data from the monotherapy with telaprevir for 15 days indicated a rapid emergence of DAA resistant variants [11]. These resistant variants can be eliminated with a combination drug regimen by pegylated interferon plus ribavirin [20]. It is clear that monotherapy with a DAA is not possible because resistance mutants appear in a few days of treatment resulting in virological rebound and treatment failure. In this scenario, researchers are looking at using combinations of DAAs targeting different viral functions for which there is no cross-resistance, as has been successfully applied at preventing resistance in HIV treatment. The rationale for an oral combination treatment for HCV is based on the present HIV treatment HAART regimen, in which multiple direct-acting antiviral drugs that target different steps of viral replication are combined to increase the amount of viral suppression (i.e. potency) and to prevent or delay the emergence of antiviral resistance (i.e. genetic barrier). DAA-resistant variants have been shown to be susceptible to peg-interferon or ribavirin, *in vitro* [11] and *in vivo* [4]; moreover, the addition of these agents has been shown to suppress the emergence of variants. Using the combination of the nucleoside analog RG7128 (high genetic barrier drug) with DAAs, such as the PIs that have a lower barrier to resistance, will reduce the chance of drug resistance emerging [73]. The INFORM-1 study confirms this benefit *in vivo* as the combination of RG7128 and danoprevir prevented resistance-associated virological breakthrough that has been reported with monotherapy with the PI. This observation was confirmed in SOC-free clinical trials combining among other DAAs, BI-201335, boceprevir, ITMN-191, and telaprevir [29–32,85–87].

Conclusions

The rapid appearance of HCV drug resistance suggests that treatment failure with monotherapy using a PI may be inevitable. Data from *in vitro* studies, early-stage clinical trials and mathematic modeling suggest that tailoring combinations of direct antiviral drugs should be beneficial to patients with CHC. However, sensitive methods to detect minor variants should be used before re-treatment of patients with the same class of antiviral drugs and the possibility of transmission of resistant viruses when these therapies become more widely available will need further consideration in the future. The resistance profiling does remain a challenge for the next generation of protease inhibitors; thus, the lessons from HIV infection treatment indicate that combinations of drugs with different mechanisms of action will be an attractive strategy for hepatitis C. The higher genetic barrier to resistance with the nucleoside analog (RG7128) polymerase inhibitors compared with either the protease or non-nucleoside polymerase inhibitors in the replicon system [52], highlights the clinical importance of the use of combinations of nucleoside analog polymerase inhibitors and protease inhibitors in future HCV therapies.

Key Points

- The HCV NS3 Protease-inhibitor associated mutations occur quickly (less than 15 days) when monotherapy is used. Monotherapy will lead to selection of resistant variants that, in turn, could produce cross-resistance to the whole class of drugs due to overlapping resistance profiles.
- Combinations of Protease Inhibitors with other class of antivirals with separate modes of action & non-overlapping resistance profile is preferable.
- The genetic barrier of the Protease Inhibitors is low, indicating that the number of mutations required for a virus to become resistant to a Protease Inhibitor is also low, and thus, the probability of selecting a mutation in the presence of the drug is high.
- There is long term persistence of HCV NS3 Protease-inhibitor associated mutations after the end of therapy.
- Cross resistance mutations exist between the different protease inhibitors.
- Ribavirin prevents viral breakthrough in combination with Pegylated Interferon and protease inhibitors.
- Detection of resistance should be done using sensitive assays that detect quasispecies at the level of 5-10% of the overall population.
- Subtyping of genotype 1 will clarify the different types of resistant variants to the PIs.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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References

[1] Zeuzem S, Berg T, Moeller B, Hinrichsen H, Mauss S, Wedemeyer H, et al. Expert opinion on the treatment of patients with chronic hepatitis C. J Viral Hepat 2009;16:75–90.
[2] Schinazi RF, Bassit L, Gavegnano C. HCV drug discovery aimed at viral eradication. J Viral Hepat 2010;17:77–90.
[3] Soriano V, Peters MG, Zeuzem S. New therapies for hepatitis C virus infection. Clin Infect Dis 2009;48:313–320.
[4] Forestier N, Reesink HW, Weegink CJ, McNair L, Kieffer TL, Chu HM, et al. Antiviral activity of telaprevir (VX-950) and peginterferon alfa-2a in patients with hepatitis C. Hepatology 2007;46:640–648.
[5] Lawitz E, Rodriguez-Torres M, Muir AJ, Kieffer TL, McNair L, Khunvichai A, et al. Antiviral effects and safety of telaprevir, peginterferon alfa-2a, and ribavirin for 28 days in hepatitis C patients. J Hepatol 2008;49: 163–169.
[6] Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, et al. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med 2009;360:1839–1850.
[7] McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 2009;360:1827–1838.
[8] Kwo PY, Lawitz EJ, McCone J, Schiff ER, Vierling JM, Pound D, et al. Efficacy of boceprevir, an NS3 protease inhibitor, in combination with peginterferon alfa-2b and ribavirin in treatment-naive patients with genotype 1 hepatitis C infection (SPRINT-1): an open-label, randomised, multicentre phase 2 trial. Lancet 2010;376:705–716.
[9] Thompson AJ, McHutchison JG. Antiviral resistance and specifically targeted therapy for HCV (STAT-C). J Viral Hepat 2009;16:377–387.
[10] Asselah T, Benhamou Y, Marcellin P. Protease and polymerase inhibitors for the treatment of hepatitis C. Liver Int 2009;29:57–67.
[11] Kieffer TL, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, et al. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. Hepatology 2007;46: 631–639.
[12] Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology 2009;138:447–462.
[13] Wohnsland A, Hofmann WP, Sarrazin C. Viral determinants of resistance to treatment in patients with hepatitis C. Clin Microbiol Rev 2007;20: 23–38.
[14] Le Guillou-Guillemette H, Vallet S, Gaudy-Graffin C, Payan C, Pivert A, Goudeau A, et al. Genetic diversity of the hepatitis C virus: impact and issues in the antiviral therapy. World J Gastroenterol 2007;13:2416–2426.
[15] Perlson E, Maday S, Fu MM, Moughamian AJ, Holzbaur EL. Retrograde axonal transport: pathways to cell death? Trends Neurosci 2010;33:335–344.
[16] Kieffer TL, Kwong AD, Picchio GR. Viral resistance to specifically targeted antiviral therapies for hepatitis C (STAT-Cs). J Antimicrob Chemother 2010;65:202–212.
[17] Rong L, Dahari H, Ribeiro RM, Perelson AS. Rapid emergence of protease inhibitor resistance in hepatitis C virus. Sci Transl Med 2010;2:30ra32.
[18] Tong X, Bogen S, Chase R, Girijavallabhan V, Guo Z, Njoroge FG, et al. Characterization of resistance mutations against HCV ketoamide protease inhibitors. Antiviral Res 2008;77:177–185.
[19] Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 2005;42:962–973.
[20] Bartels DJ, Zhou Y, Zhang EZ, Marcial M, Byrn RA, Pfeiffer T, et al. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A protease inhibitors in treatment-naive subjects. J Infect Dis 2008;198: 800–807.
[21] Kim AY, Timm J, Nolan BE, Reyor LL, Kane K, Berical AC, et al. Temporal dynamics of a predominant protease inhibitor-resistance mutation in a treatment-naive, hepatitis C virus-infected individual. J Infect Dis 2009;199: 737–741.
[22] Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. Hepatology 2008;48: 1769–1778.
[23] Nijhuis M, van Maarseveen NM, Boucher CA. Antiviral resistance and impact on viral replication capacity: evolution of viruses under antiviral pressure occurs in three phases. Handb Exp Pharmacol 2009:299–320.
[24] Cubero M, Esteban JI, Otero T, Sauleda S, Bes M, Esteban R, et al. Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. Virology 2008;370:237–245.
[25] Susser S, Welsch C, Wang Y, Zettler M, Domingues FS, Karey U, et al. Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. Hepatology 2009;50:1709–1718.
[26] Le Pogam S, Seshaadri A, Kosaka A, Chiu S, Kang H, Hu S, et al. Existence of hepatitis C virus NS5B variants naturally resistant to non-nucleoside, but not to nucleoside, polymerase inhibitors among untreated patients. J Antimicrob Chemother 2008;61:1205–1216.
[27] Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, et al. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J Biol Chem 2005;280: 36784–36791.
[28] Tong X, Chase R, Skelton A, Chen T, Wright-Minogue J, Malcolm BA. Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. Antiviral Res 2006;70:28–38.
[29] Sarrazin C, Hong J, Lim S. Incidence of virologic escape observed during ITMN-191 (R7227) monotherapy is genotype dependent, associated with a specific NS3 substitution, and suppressed upon combination with peginterferon alfa-2a/ribavirin. J Hepatol 2009;50:S350.
[30] Kukolj G, Benhamou Y, Manns MP. Bl 201335, a potent HCV NS3 protease inhibitor in treatment-naive and experienced chronic HCV genotype-1 infection: genotypic and phenotypic analysis of the NS3 protease domain. J Hepatol 2009;50:S347.
[31] Afdhal NH, Godofsky E, Dienstag J. Final phase I/II trial results for NM283, a new polymerase inhibitor for hepatitis C: antiviral efficacy and tolerance in patients with HCV-1 infection, including previous interferon failures. Hepatology 2004;40:726A.
[32] Roberts SK, Cooksley G, Dore GJ, Robson R, Shaw D, Berns H, et al. Robust antiviral activity of R1626, a novel nucleoside analog: a randomized, placebo-controlled study in patients with chronic hepatitis C. Hepatology 2008;48:398–406.
[33] Lalezari J, Gene E, Rodriguez-Torres M. Potent antiviral activity of the HCV nucleoside polymerase inhibitor R7128 with PEG-IFN and ribavirin: interim results of R7128 500mg BID for 28 days. J Hepatol 2008;48:29A.
[34] Larrey D, Benhamou Y, Lohse A. Safety, pharmacokinetics and antiviral effect of BI 207127, a novel HCV RNA polymerase inhibitor, after 5 days oral treatment in patients with chronic hepatitis C. J Hepatol 2009;50: S383–S384.
[35] Erhardt A, Deterding K, Benhamou Y, Reiser M, Forns X, Pol S, et al. Safety, pharmacokinetics and antiviral effect of BILB 1941, a novel hepatitis C virus RNA polymerase inhibitor, after 5 days oral treatment. Antivir Ther 2009;14:23–32.
[36] Lawitz E, Sulkowski M, Jacobson IM. Safety, tolerability and antiviral activity of MK-7009, a novel inhibitor of the hepatitis C virus NS3/4A protease, in patients with chronic HCV genotype 1 infection. Hepatology 2008;48:403A.
[37] Pasquinelli C, Eley T, Villegas C. Safety, tolerability, pharmacokinetics and antiviral activity following single and multiple dose administration of BMS-650032, a novel HCV NS3 inhibitor, in subjects with chronic genotype 1 HCV infection. Hepatology 2009;50:225.
[38] Hotho M, deBruijne J, O'Farrell A. Accelerated clinical trial design to assess the safety, tolerability and anti-viral activity of PHX1766, a novel HCV NS4/4A protease inhibitor, in healthy volunteers and chronic hepatitis C patients. Hepatology 2009;50:1576.
[39] Seiwert S, Hong J, Lim S. Sequence veriation of NS3/4A in HCV replicons exposed to ITMN-191 concentrations encompassing those likely to be achieved following clinical dosing. J Hepatol 2007;46:244A.
[40] Manns MP, Reesink HW, Moreno C. OPERA-1 trial: interim analysis of safety and antiviral activity of TMC435 in treatment naive genotype 1 HCV patients. J Hepatol 2009;50:S7.
[41] Neumann GA, Mazarico E. Planetary science. Seeing the missing half. Science 2009;323:885–887.
[42] Domingo E, Gomez J. Quasispecies and its impact on viral hepatitis. Virus Res 2007;127:131–150.
[43] Franco S, Parera M, Aparicio E, Clotet B, Martinez MA. Genetic and catalytic efficiency structure of an HCV protease quasispecies. Hepatology 2007;45:899–910.
[44] Ruiz-Jarabo CM, Arias A, Molina-Paris C, Briones C, Baranowski E, Escarmis C, et al. Duration and fitness dependence of quasispecies memory. J Mol Biol 2002;315:285–296.

[45] Rong L, Perelson AS. Treatment of hepatitis C virus infection with interferon and small molecule direct antivirals: viral kinetics and modeling. Crit Rev Immunol 2010;30:131–148.
[46] Richman DD. The implications of drug resistance for strategies of combination antiviral chemotherapy. Antiviral Res 1996;29:31–33.
[47] Richman DD. The impact of drug resistance on the effectiveness of chemotherapy for chronic hepatitis B. Hepatology 2000;32:866–867.
[48] Sarrazin C, Kieffer TL, Bartels D, Hanzelka B, Muh U, Welker M, et al. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 2007;132: 1767–1777.
[49] Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, McDade H. Viral dynamics in hepatitis B virus infection. Proc Natl Acad Sci USA 1996;93: 4398–4402.
[50] Bartenschlager R, Lohmann V. Replication of the hepatitis C virus. Baillieres Best Pract Res Clin Gastroenterol 2000;14:241–254.
[51] Shimakami T, Welsch C, Yamane D, McGivern D, Yi M, Zeuzem S, et al. Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. Gastroenterology 2011;46: 667–675.
[52] Verbinnen T, Van Marck H, Vandenbroucke I, Vijgen L, Claes M, Lin TI, et al. Tracking the evolution of multiple in vitro hepatitis C virus replicon variants under protease inhibitor selection pressure by 454 deep sequencing. J Virol 2010;84:11124–11133.
[53] Curry S, Qiu P, Tong X. Analysis of HCV resistance mutations during combination therapy with protease inhibitor boceprevir and PEG-IFN alpha-2b using TaqMan mismatch amplification mutation assay. J Virol Methods 2008;153:156–162.
[54] Luber AD. Genetic barriers to resistance and impact on clinical response. MedGenMed 2005;7:69.
[55] McCown MF, Rajyaguru S, Kular S, Cammack N, Najera I. GT-1a or GT-1b subtype-specific resistance profiles for hepatitis C virus inhibitors telaprevir and HCV-796. Antimicrob Agents Chemother 2009;53:2129–2132.
[56] Hang JQ, Yang Y, Harris SF, Leveque V, Whittington HJ, Rajyaguru S, et al. Slow binding inhibition and mechanism of resistance of non-nucleoside polymerase inhibitors of hepatitis C virus. J Biol Chem 2009;284: 15517–15529.
[57] McCown MF, Rajyaguru S, Le Pogam S, Ali S, Jiang WR, Kang H, et al. The hepatitis C virus replicon presents a higher barrier to resistance to nucleoside analogs than to nonnucleoside polymerase or protease inhibitors. Antimicrob Agents Chemother 2008;52:1604–1612.
[58] Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
[59] Shaw T, Locarnini SA. Preclinical aspects of lamivudine and famciclovir against hepatitis B virus. J Viral Hepat 1999;6:89–106.
[60] Asselah T. Genetic polymorphism and response to treatment in chronic hepatitis C: the future of personalized medicine. J Hepatol 2010;52: 452–454.
[61] Pawlotsky JM, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. Gastroenterology 2007;132:1979–1998.
[62] Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature 2010;465:96–100.
[63] Johnson CL, Gale Jr M. CARD games between virus and host get a new player. Trends Immunol 2006;27:1–4.
[64] Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci USA 2005;102: 2992–2997.
[65] Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 2005;437:1167–1172.
[66] Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, et al. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature 2003;426:186–189.
[67] Benhamou Y, Moussalli J, Ratziu V. Results of a prove of concept study (C210) of telaprevir monotherapy and in combination with peginterferon alfa-2a and ribavirin in treatment-naive genotype 4 HCV patients. J Hepatol 2009;50:S6.
[68] Courcambeck J, Bouzidi M, Perbost R, Jouirou B, Amrani N, Cacoub P, et al. Resistance of hepatitis C virus to NS3-4A protease inhibitors: mechanisms of drug resistance induced by R155Q, A156T, D168A and D168V mutations. Antivir Ther 2006;11:847–855.
[69] Zhou Y, Bartels DJ, Hanzelka BL, Muh U, Wei Y, Chu HM, et al. Phenotypic characterization of resistant Val36 variants of hepatitis C virus NS3-4A serine protease. Antimicrob Agents Chemother 2008;52:110–120.
[70] Prongay AJ, Guo Z, Yao N, Pichardo J, Fischmann T, Strickland C, et al. Discovery of the HCV NS3/4A protease inhibitor (1R,5S)-*N*-[3-amino-1-(cyclobutylmethyl)-2,3-dioxopropyl]-3-[2(*S*)-[[[(1,1-dimethylethyl)amino]carbonyl]amino]-3,3-dimethyl-1-oxobutyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexan-2(*S*)-carboxamide (Sch 503034) II. Key steps in structure-based optimization. J Med Chem 2007;50:2310–2318.
[71] Bae A, Sun SC, Qi X, Chen X, Ku K, Worth A, et al. Susceptibility of treatment-naive hepatitis C virus (HCV) clinical isolates to HCV protease inhibitors. Antimicrob Agents Chemother 2010;54:5288–5297.
[72] Lenz O, Verbinnen T, Lin TI, Vijgen L, Cummings MD, Lindberg J, et al. In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. Antimicrob Agents Chemother 2010;54:1878–1887.
[73] Cummings MD, Lindberg J, Lin TI, de Kock H, Lenz O, Lilja E, et al. Induced-fit binding of the macrocyclic noncovalent inhibitor TMC435 to its HCV NS3/NS4A protease target. Angew Chem Int Ed Engl 2010;49: 1652–1655.
[74] Durant J, Clevenbergh P, Halfon P, Delgiudice P, Porsin S, Simonet P, et al. Drug-resistance genotyping in HIV-1 therapy: the VIRADAPT randomised controlled trial. Lancet 1999;353:2195–2199.
[75] Zhou Y, Muh U, Hanzelka BL, Bartels DJ, Wei Y, Rao BG, et al. Phenotypic and structural analyses of hepatitis C virus NS3 protease Arg155 variants: sensitivity to telaprevir (VX-950) and interferon alpha. J Biol Chem 2007;282:22619–22628.
[76] Qi X, Bae A, Liu S, Yang H, Sun SC, Harris J, et al. Development of a replicon-based phenotypic assay for assessing the drug susceptibilities of HCV NS3 protease genes from clinical isolates. Antiviral Res 2009;81:166–173.
[77] McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, et al. Telaprevir for previously treated chronic HCV infection. N Engl J Med 2010;362:1292–1303.
[78] Zeuzem S, Sulkowski M, Zoulim F. Long-term follow-up of patients with chronic hepatitis C treated with telaprevir in combination with peginterferon alfa-2a and ribavirin: interim analysis of the EXTEND study. Hepatology 2010;52:A227.
[79] Vierling JM, Kwo PY, Lawitz E, McCone J, Schiff ER, Pound D, et al. Frequencies of resistance-associated amino acid variants following combination treatment with boceprevir plus PEGINTRON (PegInterferon Alfa-2b)/ribavirin in patients with chronic hepatitis C (CHC), genotype 1 (G1). Hepatology 2010;52:A801.
[80] Qiu P, Sanfiorenzo V, Curry S, Guo Z, Liu S, Skelton A, et al. Identification of HCV protease inhibitor resistance mutations by selection pressure-based method. Nucleic Acids Res 2009;37:e74.
[81] Gaudieri S, Rauch A, Pfafferott K, Barnes E, Cheng W, McCaughan G, et al. Hepatitis C virus drug resistance and immune-driven adaptations: relevance to new antiviral therapy. Hepatology 2009;49:1069–1082.
[82] Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, et al. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting $CD4^+$ T cells. Nat Med 2003;9:727–728.
[83] Palmer S, Maldarelli F, Wiegand A, Bernstein B, Hanna GJ, Brun SC, et al. Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy. Proc Natl Acad Sci USA 2008;105:3879–3884.
[84] Halfon P, Riflet H, Renou C, Quentin Y, Cacoub P. Molecular evidence of male-to-female sexual transmission of hepatitis C virus after vaginal and anal intercourse. J Clin Microbiol 2001;39:1204–1206.
[85] Reesink HW, Fanning GC, Farha KA, Weegink C, Van Vliet A, Van't Klooster G, et al. Rapid HCV-RNA decline with once daily TMC435: a phase I study in healthy volunteers and hepatitis C patients. Gastroenterology 2010;138: 913–921.
[86] Gane EJ, Roberts SK, Stedman CA, Angus PW, Ritchie B, Elston R, et al. Oral combination therapy with a nucleoside polymerase inhibitor (RG7128) and danoprevir for chronic hepatitis C genotype 1 infection (INFORM-1): a randomised, double-blind, placebo-controlled, dose-escalation trial. Lancet 2010;376:1467–1475.
[87] Zeuzem S, Buggisch P, Agarwal K, Manns MP, Marcellin P, Foster GR, et al. Dual, triple, and quadruple combination treatment with a protease inhibitor (GS-9256) and a polymerase inhibitor (GS-9190) alone and in combination with ribavirin (RBV) or PegIFN/RBV for up to 28 days in treatment naive, genotype 1 HCV subjects. Hepatology 2010;52:Oral LB-1.